

Activity of Orange (*Citrus sinensis*) and Lemon (*Citrus limon*) juice and oil on different bacteria that cause wound infection

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Abstract

The antibacterial activity of *Citrus sinensis* and *Citrus limon* was evaluated on bacterial strains. Juice and oil were used by agar disc and well diffusion. The antibacterial effects of Citrus oil were found to be effective using disc method among all strains, except *Clostridium* spp, which was found no inhibition with *Citrus sinensis* oil. However, *Clostridium* spp with *Citrus limon* oil had inhibitory zone (7 ± 0 mm), while the remainder of organisms, it showed moderate zones of inhibition ranging from 6-9.66mm in diameter. By well diffusion method, it showed highest zone of inhibition on *S. aureus*, which was 12mm in diameter, while on the other organisms with Citrus it showed little or no zones of inhibition ranging from 0-6mm in diameter. The juice from *C. limon* juice exhibited inhibitory activities against bacteria in using the disc method except for *S. pyogenes*, which was found no inhibition, while other organisms, it showed moderate zones of inhibition ranging from 6.66-12mm in diameter. *Citrus sinensis* juice showed no inhibition against bacteria. The results obtained in this study suggested that *Citrus limon* had overall better antibacterial activity compared to *Citrus sinensis*.

Keywords: *Citrus sinensis*, *Citrus limon*, Disc Diffusion, Well Diffusion

1. Introduction

Citrus is one of the most important fruit grown in many countries of the world. Orange and lemon are an important medicinal plant of the family Rutaceae. They are cultivated for long time age, for dietary intake, as well as to be used as herbal medicines. This is mainly for their content of alkaloid which is present in all parts of the plant (edible part, leaves, stem, roots, or flower) of citrus against clinically significant bacterial strains [9]. Usually in industries, orange fruits are processed alone, while peels are considered to be redundant. However, the orange peel contain a large quantity of nutrients and other important chemicals such as flavonoid, glycosides, coumarins, sitosterols, glycosides and volatile oils that can be used in food and medicine [19]. In addition, the fiber of citrus fruit also contains bioactive compounds, such as polyphenols. The most medically significant use of orange is to treat scurvy disease, due to high content of vitamin C in orange fruit. [1, 10]. In addition, many studies reported its antioxidant and antibacterial effects of the juice and edible part of lemon and orange [6]. Currently, drug resistance issue against commonly used antibiotic is the main problem to treat bacterial related diseases. This is due to the ability of bacteria to transmit and acquire resistance to those drugs [13]. To overcome this problem, researchers, scientists, pharmacological companies and industries are in rush to find new alternatives. One of their investigations is on how to use lemon and orange contents as an antibacterial substance. Throughout the mankind history, several infectious diseases have been known to be treated with herbal medicine, because of medicine content of herbal extracts [16, 17]. This opened the door for prominence of discovering in this field. This study look into the possibilities of using lemon and orange to treat wound infections. It aims to

evaluate antimicrobial activity of citrus on selected pathogens obtained from wound infections.

2. Materials and Methods

1. Materials

Equipment: Autoclave, Electronic weighing balance, Hot-air oven, Refrigerator,

2. Compounds

Orange oil (Korean company), lemon oil (Purchased from local mall in Malaysia) and orange and lemon juice (Purchased from local mall)

3. Culture media

Mueller-Hinton agar, Nutrient agar, chocolate agar and blood agar.

4. Antibiotic disc

Amoxicillin-Clavulanic acid, Gentamicin, Streptomycin and Tetracycline.

2.1 Citrus oil and juice

Citrus sinensis oil was commercially purchased from Korea and *Citrus limon* oil is purchased from Malaysia from local mall. On the other hand, procurement of lemon (*Citrus limon*) and oranges (*Citrus sinensis*) juice were purchased from local mall. The juice is squeezed from fresh fruit and stored in glass bottles in refrigerator.

2.2 Source of Microbial Cultures

Cultures were obtained from the Medical Microbiology and Parasitology Research Laboratory of HUKM, Hospital

Universiti Kebangsaan Malaysian. They include: three strains of Gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus spp*, and *Clostridium spp*); three strains of Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*). The bacterial cultures were maintained in their respective agar slants at 4°C throughout the course of the study and used as stock cultures.

2.3 Inoculum Standardization

The test organisms were sub cultured on fresh plates of Mueller-Hinton medium and incubated for 24 h at 37°C. The agar plates were stored at 4°C until required. Overnight cultures from these plates were suspended into normal saline for antimicrobial assays. They were adjusted to a turbidity matching the 0.5 McFarland standard.

Antimicrobial Activity of Citrus Oil and Juice from Orange and Lemon:

2.4 Disc Diffusion Method

Disc diffusion method for antimicrobial susceptibility testing was performed according to the standard method by “reference [3]” to assess the presence of antibacterial activities of the Citrus oil and juice. A bacterial suspension (which has been adjusted to 0.5 McFarland standard), was used to lawn Muller Hinton agar plates evenly using a sterile swab. The plates were dried for 15 minutes at room temperature and then used for the sensitivity test. The discs which had been impregnated with a series of plant extracts were placed on the Mueller- Hinton agar surface. Each test plate includes of two discs, one negative control and one positive control. The positive control used was a standard commercial antibiotic disc. The standard antibiotic discs were ampicillin, tetracycline, streptomycin and gentamicin. For the negative control, distilled water and Tween 20 were used. Besides the controls, each plate had three treated discs placed about equidistance to each other. The plate was then incubated at 37°C for 18 to 24 hours depending on the species of bacteria used in the test. After the incubation, the plates were examined for inhibition zone. The inhibition zone were then measured using calipers and recorded. The tests were repeated three times to ensure reliability.

2.5 Well Diffusion Method

Screening of citrus juice and oil for antimicrobial activity was done by the agar well diffusion method [22] using microorganism cell suspension at 0.5 McFarland standards. Each labelled medium plate was uniformly categorized with a test organism by means of sterile swab rolled in suspension and streaked on the plate surface. Wells of 5mm in diameter and 2cm apart were punched on the culture medium. Approximately 20µl of each extract was inoculated onto wells were made in the spread plate culture of each microbial isolates (The plates were carried out in triplicates). All plate of the tested organisms was then allowed to incubate at 37°C for overnight. After 24 h of incubation, each extract was noted for zone of inhibition for all isolates. The diameters of the zone of inhibitions were measured by measuring scale in millimeter (mm).

3. Results

3.1 Overall comparison for disc diffusion method

“Figure 1” indicates that all the 3 extracts showed a various

wide spectrum activity against the test organisms used disc in the study. However, it was found that lemon juice had maximum inhibitory action against all the test organisms except *Streptococcus pyogenes* which was unaffected by lemon juice. Also it was found that lemon and sweet orange oil had minimum inhibition against all the test organisms especially with *Clostridium spp* which was unaffected by sweet orange oil.

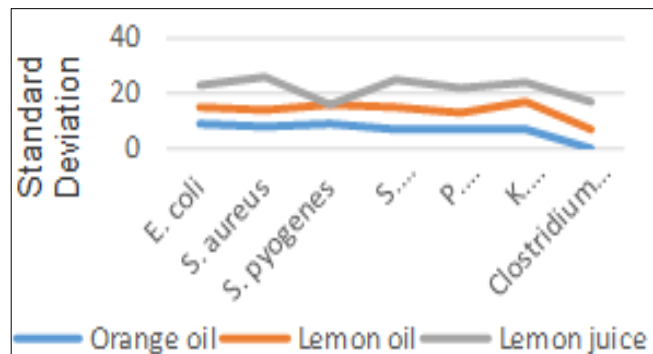


Fig 1: Graph showing diameter of zone of inhibition (in mm) with respect to the compounds against the test organisms using disc

3.2 Overall comparison for well diffusion method

“Figure 2” indicates that all the 3 compounds showed a various wide spectrum activity against the test organisms used well in the study. It was found that lemon juice had maximum inhibitory action against all the test organisms except *Streptococcus pyogenes* which was unaffected by lemon juice. Moreover it was found that lemon oil has shown inhibition zones with *Streptococcus pneumoniae* and *Clostridium spp*. Also the sweet orange oil has shown inhibition zones with *Streptococcus pyogenes* and *Staphylococcus aureus*.

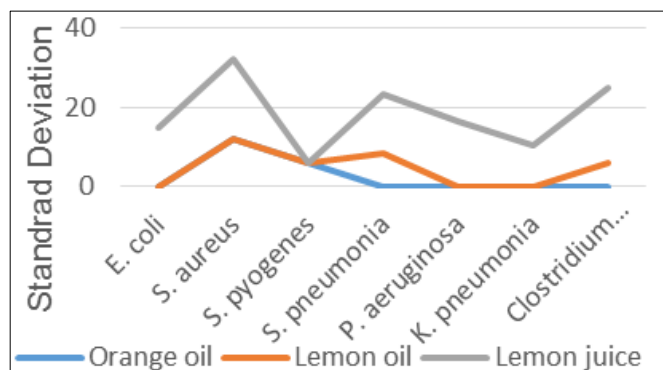


Fig 2: Graph showing diameter of zone of inhibition (in mm) with respect to the 3 compounds against the test organisms using well

3.3 Positive Control

The positive control used was a standard commercial antibiotic disc. The standard antibiotic discs were ampicillin, tetracycline, streptomycin and gentamicin which have inhibition zone against most of bacteria strains used (Table.1).

3.4 Antibacterial Activity of Citrus Oil

Antimicrobial activity of orange and lemon oil was assessed against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Clostridium spp* and *Streptococcus*

pyogenes. The diameters of the inhibition zone are presented in Table. 2, Table. 3, Table. 4 and Table. 5.

3.5 Antibacterial Activity of Orange Oil

Orange oil disc diffusion method showed inhibition against all the Gram positive and Gram negative bacteria used in the present study except *Clostridium spp* which has no inhibition against orange oil disc and well method, the maximum inhibition zone was observed in *Streptococcus pyogenes* and minimum inhibition zone in *Streptococcus pneumoniae* Table. 2. By well method, only have inhibition zone against with *Staphylococcus aureus*, *Streptococcus pyogenes* was observed high and *Clostridium spp* shows decreased zone of inhibition Table.3.

3.6 Antibacterial Activity of Lemon Oil

Lemon oil disc showed inhibition against all the test organisms (Gram positive and Gram negative) with maximum zone of inhibition about 10mm against *Klebsiella pneumoniae* and minimum zone with *Escherichia coli* and *Pseudomonas aeruginosa* Table. 4. For well method, inhibition zone was observed only with *Streptococcus pneumoniae* and *Clostridium spp* Table.5.

3.7 Antibacterial Activity of Citrus Juice

Bacterial activity of orange and lemon juice was examined against the wound causing bacteria as mentioned above. The results are presented in Table. 6 and Table. 7 in terms of test organisms used and the diameter of zone of inhibition.

3.8 Antibacterial Activity of Lemon Juice

Lemon juice showed inhibition against all the test organisms (Gram positive and Gram negative) with disc a maximum zone of inhibition against *Staphylococcus aureus* and minimum zone of inhibition against *Streptococcus pyogenes* Table 4.6. By well method maximum zone of inhibition against *Staphylococcus aureus* and minimum zone of inhibition against *Streptococcus pyogenes* Table 4.7. Lemon juice thus showed a wide spectrum activity against all the Gram positive and Gram negative used in the present study.

3.9 Antibacterial Activity of Orange Juice

Orange juice showed no inhibition against all the test organisms (Gram positive and Gram negative) with disc and well diffusion method. On the other hand, orange juice thus showed ineffective against all the Gram positive and Gram negative used in the present study (data not shown).

Table 1: Positive control by standard commercial antibiotic disc

Organisms	Am	T	S	G
<i>Escherichia coli</i>	25mm	20mm	30mm	R
<i>Staphylococcus aureus</i>	10mm	R	20mm	25mm
<i>Streptococcus pyorogene</i>	R	30mm	R	R
<i>Streptococcus pneumoniae</i>	10mm	R	25mm	R
<i>Pseudomonas aeruginosa</i>	R	R	R	20mm
<i>Klebsiell pneumoniae</i>	R	R	R	35mm
<i>Clostridium spp</i>	5mm	R	R	25mm

R: Resistant, Am: Ampicillin, T: Tetracycline, S: Streptomycin, G: Gentamicin

Table 2: Showing diameter of zone of inhibition (in mm) of orange oil (disc method) against clinical isolates from wound infection

Organisms	Diameter of Zone of inhibition with orange oil (in mm)			Me an± S.D (in mm)
<i>Escherichia coli</i>	8	9	9	8.66±0.75
<i>Staphylococcus aureus</i>	6	8	8	7.33±1.15
<i>Streptococcus. Pyogenes</i>	9	10	8	9±1.0
<i>Streptococcus pneumonia</i>	6	8	6	6.6±1.1
<i>Pseudomonas aeruginosaji</i>	7	7	7	7±0.0
<i>Klebsiella pneumonia</i>	7	7	7	7±0.0
<i>Clostridium spp</i>	0	0	0	0.0

Table 3: Showing diameter of zone of inhibition (in mm) of orange oil (well method) against clinical isolates from wound infection

Organisms	Diameter of Zone of inhibition with orange oil (in mm)			Me an± S.D (in mm)
<i>Escherichia coli</i>	0	0	0	0.0
<i>Staphylococcus aureus</i>	11	10	12	12±02.64
<i>Streptococcus pyogenes</i>	6	6	6	6±0.0
<i>Streptococcus pneumonia</i>	0	0	0	0.0
<i>Pseudomonas aeruginosa</i>	0	0	0	0.0
<i>Klebsiella pneumonia</i>	0	0	0	0.0
<i>Clostridium spp</i>	0	0	0	7±0.0

Table 4: Showing diameter of zone of inhibition (in mm) with resp to lemon oil using disc

Organism	Diameter of Zone of inhibition with lemon oil (in mm)			Me an± S.D (in mm)
<i>Escherichia coli</i>	6	6	6	6±0.0
<i>Staphylococcus aureus</i>	7	7	6	6.66±0.57
<i>Streptococcus pyogenes</i>	7	7	7	7±0.0
<i>Streptococcus pneumonia</i>	7	8	8	7.66±0.57
<i>Pseudomonas aeruginosa</i>	10	5	6	6±1.0
<i>Klebsiella pneumonia</i>	7	10	9	9.66±0.57
<i>Clostridium spp</i>	6	7	7	7±0.0

Table 5: Showing diameter of zone of inhibition (in mm) with respect to lemon oil using well

Organisms	Diameter of Zone of inhibition with lemon oil (in mm)			Me an± S.D (in mm)
<i>Escherichia coli</i>	0	0	0	0.0
<i>Staphylococcus aureus</i>	0	0	0	0.0
<i>Streptococcus pyogenes</i>	0	0	0	0.0
<i>Streptococcus pneumonia</i>	10	8	7	8.33±1.52
<i>Pseudomonas aeruginosa</i>	0	0	0	0.0
<i>Klebsiella pneumonia</i>	0	0	0	0.0
<i>Clostridium spp</i>	6	6	6	6±0.0

Table 6: Showing diameter of zone of inhibition (in mm) with respect to lemon juice using disc

Organisms	Diameter of Zone of inhibition with lemon juice (in mm)			Me an± S.D (in mm)
<i>Escherichia coli</i>	9	8	8	8.33±0.57
<i>Staphylococcus aureus</i>	12	12	12	12±0.0
<i>Streptococcus pyogenes</i>	0	0	0	0.0
<i>Streptococcus pneumonia</i>	11	11	9	10.33±1.15
<i>Pseudomonas aeruginosa</i>	7	10	8	8.33±1.52
<i>Klebsiella pneumonia</i>	6	7	7	6.66±0.57
<i>Clostridium spp</i>	10	9	9	9.33±0.57

Table 7: Showing diameter of zone of inhibition (in mm) with respect to lemon juice using well

Organisms	Diameter of Zone of inhibition with lemon juice (in mm)			Me an± S.D (in mm)
<i>Escherichia coli</i>	15	14	15	14.66±0.57
<i>Staphylococcus aureus</i>	18	21	21	20±1.73
<i>Streptococcus pyogenes</i>	0	0	0	0.0
<i>Streptococcus pneumonia</i>	16	15	14	15±1
<i>Pseudomonas aeruginosa</i>	17	15	17	16.33±1.15
<i>Klebsiella pneumonia</i>	11	11	9	10.33±1.15
<i>Clostridium spp</i>	20	21	16	19±2.64

4. Discussion

4.1 Positive Control

The positive control indicates that all the antibiotics tested such as ampicillin, tetracycline, streptomycin and gentamicin showed inhibition against Gram positive and Gram negative bacteria. However it was found that gentamicin was most effective against most of the organisms used in the present study.

4.2 Negative Control

Negative control by using distilled water and Tween 20 showed no inhibition against all the test organisms.

4.3 Antibacterial Activity of Citrus Oil Using Disc Method

The oils of *Citrus sinensis* and *Citrus limon* were found to be equally effective against both groups of bacteria (Gram positive and negative), except *Clostridium spp* which was found no inhibition with *Citrus sinensis* oil. However, *Clostridium spp* with *Citrus limon* oil had inhibitory zone (7±0mm). On the other hand, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa* with *Citrus sinensis* oil showed higher inhibitory zones than *Citrus limon* oil (8.66±0.75), (7.33±1.15), (9±1) and (7±0) respectively. But *Streptococcus pneumonia* and *Klebsiella pneumoniae* with *Citrus limon* oil have shown higher inhibitory zones than *Citrus sinensis* oil (7.66±0.57) and (9.66±0.57) respectively. This result suggests the effect of antibacterial activity of *Citrus sinensis* and *Citrus limon* oil against clinical isolates, similar to previous finding that reported orange oil using well diffusion method [15]. Another study with orange and lemon oil, had higher inhibitory zones compared to this study [20]. Previous studies

indicated that *S. aureus* presented a variable sensitivity to different citrus EOs species [4].

4.4 Antibacterial Activity of Citrus Juice

The juice from *Citrus limon* exhibited inhibitory activities against Gram positive and Gram negative bacteria in using the disc diffusion method except for *Streptococcus pyogenes*. With limon juice, maximum inhibition zone was observed with *Staphylococcus aureus* (12±0) and minimum inhibition zone with *Klebsiella pneumoniae* (6.66±0.57). Our result correcte with [2]. Previous studies have shown lemon extract to have significant antimicrobial activity against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* [7]. However *Citrus sinensis* extract showed no inhibition shown activities against Gram positive and Gram negative using the disc diffusion method, similar as other studies [5, 8, 12].

4.5 Antibacterial Activity of Citrus Oil Using Well Method

Antibacterial activities from *Citrus sinensis* and *Citrus limon* oil were carried out against Gram positive and Gram negative bacteria using the well diffusion method. The *Citrus sinensis* showed no inhibition zones against both groups of bacteria (Gram positive and negative) except for *Staphylococcus aureus*, which has moderate inhibition zones (12±2.64) and *Streptococcus pyogenes* minimum inhibition zones (6±0). For *Citrus limon* oil, no inhibition zones against both groups of bacteria (Gram positive and negative) except *Streptococcus pneumonia* (8.33±1.52) and *Clostridium spp* (6±0). Studies by "reference [15]" has showed orange oil had antibacterial activity on *Staphylococcus aureus*. Another study has shown

high and moderate inhibition zones with *Citrus sinensis* and *Citrus limon* oil ^[20].

4.6 Antibacterial Activity of Citrus Juice

The juice from *Citrus limon* juice exhibited inhibitory activities against Gram positive and Gram negative using the well diffusion method except with *Streptococcus pyogenes*. Furthermore, with limon juice the maximum inhibition zone was observed in *Staphylococcus aureus* (20±1.73) and the minimum inhibition zone was observed in *Klebsiella pneumoniae* (10.33±1.15). Previous studies also indicated *C.limon* had good inhibition zone against *Staphylococcus aureus* ^[2]. Moreover, investigation on antimicrobial properties of lemon has shown lemon has significant antimicrobial activity against *Staphylococcus aureus* *Klebsiell pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa*, especially for *S. aureus* ^[7]. In this study, *Citrus sinensis* (orange juice) showed no inhibition against the bacteria tested. This result is similar to another study which indicated that *Citrus sinensis* had no inhibition activities against Gram positive and Gram negative using the well diffusion method ^[5, 8, 12]. In contrast, there are findings on positive antibacterial activity of orange against bacteria ^[11, 14]. Lemon played a significant role as antimicrobial agents as they are natural, cheap, safe, and as an alternative therapy to common antibiotic. Furthermore, difference between *Citrus sinensis* and *Citrus limon* antibacterial effect could be due to difference in the phytochemical components in various part of the plant or may be also due to the Citrus used and/or environmental factors or difference in the genotypes of the Citrus plant ^[21]. Also for the various sensitivity of the Gram-negative bacteria compared to that of Gram-positive bacteria may be because differences in their cell wall components. Gram-positive bacteria contain an outer peptidoglycan layer, which acts important role as a permeability barrier, whereas Gram-negative bacteria have an outer phospholipid membrane ^[18].

5. Conclusion

The present study is to investigate the antibacterial activity of orange oil, lemon oil, orange juice and lemon juice against bacteria using disc method and well method. The overall result clearly showed that disc method offered a more efficient way to investigate antibacterial effect of Citrus. And for the two different type of citrus content tested in juice and oil solutions, it was observed juice had better inhibition zone compared to oil base. Lastly, it was found lemon juice (*Citrus limon*) had overall better antibacterial activity compared to orange juice (*Citrus sinensis*).

6. Acknowledgments

1. The mechanism of action of extract citrus juice/oil and their major components at high level should be investigated.
2. Isolation and purification of major components from extract citrus juice/ oil should be performed.
3. Safety studies for using extract citrus juice/oil in greater concentration should be determined.

7. References

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